Specificity of different isoforms of protein phosphatase-2A and protein phosphatase-2C studied using site-directed mutagenesis of HMG-CoA reductase

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Received 25 May 1997

Abstract We have expressed the catalytic domain of Chinese hamster HMG-CoA reductase, and 13 point mutations involving the region around the single phosphorylation site for AMP-activated protein kinase. After phosphorylation, these were used to test the specificity of isoforms of protein phosphatase-2A [bovine $PP2A_C$ (catalytic subunit) and $PP2A_1$ (ABC heterotrimer)] and protein phosphatase-2C (human α; mouse α, β1, β2, β3, β4, β5). $PP2A_1$ had > 50-fold higher activity for HMG-CoA reductase variants than $PP2A_C$, but their relative selectivity for different variants was similar. Although the specificities of PP2A and PP2C were distinct, no dramatic differences in selectivity were observed between different PP2C isoforms.

Key words: Protein phosphatase-2A; Protein phosphatase-2C; dephosphorylation; HMG-CoA reductase; AMP-activated protein kinase; Site-directed mutagenesis

1. Introduction

The serine/threonine-specific protein phosphatases (PP) in eukaryotic cells can be divided into two distinct families which are not related in sequence: (i) the PPP family, including the PP1, PP2A and PP2B subfamilies; (ii) the PPM family, including the PP2C subfamily [1]. PP2A holoenzymes comprise a 36 kDa catalytic (C) subunit complexed with an A subunit (PR65) and one of several B subunits (e.g. PR55) [2]. The enzyme is thought to be an ABC heterotrimer in vivo, although free AC dimer may also exist. Two isoforms of PP2C (α and β) have been defined by amino acid [3] and DNA sequencing [4–7]. In addition, mouse PP2Cβ exists as five variants (β1 to β5) which appear to be generated by alternative splicing [8,9]. Unlike PP2A, PP2Cs are monomers, at least in mammals.

By contrast with protein kinases [10], much less is known about how protein phosphatases recognize their target proteins. Some features of the specificity of members of the PP1, PP2A and PP2C subfamilies have been deduced using synthetic peptide substrates [11–13]. However, it has proved difficult to correlate recognition determinants derived from synthetic peptide studies with the specificity of these phosphatases for protein substrates. One reason might be that protein phosphatases recognize secondary or tertiary structure rather than simple primary sequence determinants. Therefore specificity studies performed using mutations of a native protein might be more informative than those performed using synthetic peptides. We have recently expressed the catalytic domain of Chinese hamster 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in *Escherichia coli*, and studied the specificity of rat AMP-activated protein kinase (AMPK) using the wild-type protein and several point mutations at or near the single phosphorylation site [14]. We have now used the phosphorylatable variants to study the specificity of the free catalytic subunit and heterotrimer forms of PP2A, human PP2Cα, and the α, β1, β2, β3, β4 and β5 isoforms of mouse PP2C.

2. Material and methods

2.1. Materials

Histidine-tagged HMG-CoA reductase mutants were expressed in *E. coli* and purified to homogeneity [14]. AMPK was purified from rat liver as far as the gel filtration step [15] and was a mixture of the α1 and α2 isoforms [16]. Human PP2C-a was expressed and purified from *E. coli* [17]. PP2A catalytic subunit and ABC heterotrimer forms were purified from bovine heart and rabbit skeletal muscle [18].

Expression constructs for mouse PP2Cs were made from the plasmids pKMC-PP2Cα [7], pKMC-MPP β-1 and β-2 [9] and TK-3, 4 and 5 [8]. NcoI-EcoRI fragments were cloned in the correct reading frame into pGEX-2T. The plasmids were used to transform *E. coli* DH5α, expression being in 300 ml cultures for 5 h at 30°C in 100 μM isopropylthiogalactoside. Cells were collected by centrifugation and lysed by sonication in 3 ml ice-cold phosphate-buffered saline (PBS) containing 1 μM phenylmethane sulphonylfluoride. Triton X-100 (1%, v/v) was added and the suspension centrifuged (10 000 × *g*; 10 min). Glutathione-agarose (200 μl, 50% (v/v) suspension) was added to the supernatant, incubated in a rotary shaker for 15 min at 4°C, and the beads packed into a column. The column was washed with 12 ml of PBS plus Triton X-100 (1%, v/v), then 4 ml of 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, then 4 ml of the same buffer plus 2.5 mM $CaCl_2$ (cleavage buffer). The glutathione-agarose was removed from the column, incubated in 300 μl cleavage buffer containing thrombin (Sigma, 10 μg/ml) for 1 h at 25°C, and the suspension centrifuged (200 × *g*; 10 s). The PP2C isoforms in the supernatant were stored at −70°C.

2.2. Preparation of phosphosubstrates, protein phosphatase assays and other procedures

HMG-CoA reductase (200 μg/ml), AMPK (0.8 U/ml), AMP (200 μM), $MgCl_2$ (5 mM) and [γ-^{32}P]ATP (200 μM, 500–1000 dpm/pmol) were incubated for 30 min at 30°C. ATP was removed by centrifuging the sample through a Sephadex G-50 Nick Spin column equilibrated in 1 M imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.5. Protein

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Abbreviations: AMPK, AMP-activated protein kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PP1, protein phosphatase-1; $PP2A_C$, protein phosphatase-2A catalytic subunit; $PP2A_1$, protein phosphatase-2A ABC heterotrimer; PP2C, protein phosphatase-2C; PBS, phosphate-buffered saline; Sp, phosphoserine; Tp, phosphothreonine

PII S0014-5793(97)00712-6

phosphatase assays (30 μl) contained 10 μl of protein phosphatase in Tris/HCl (50 mM, pH 7.5), EGTA (0.1 M), 2-mercaptoethanol (0.03%, v/v), bovine serum albumin (0.33 mg/ml), Brij-35 (0.01%, w/v). The reaction was started by adding [^{32}P]HMG-CoA reductase to 2.6 μM final, and after incubation at 30°C the reaction was stopped by adding 200 μl of trichloroacetic acid (20%, w/v). The precipitate was removed by centrifugation (14 000 × *g*, 3 min) and 200 μl of the supernatant was counted in 1 ml of scintillant. At least 3 time points were measured to ensure that initial rates were being measured.

Protein concentration was measured by the method of Bradford [19]. SDS-polyacrylamide gel electrophoresis was by the method of Laemmli [20].

3. Results

3.1. Characterization of phosphorylated HMG-CoA reductase and protein phosphatases

All protein phosphatases used in this study were homogeneous by SDS-PAGE (Fig. 1; not shown for mouse PP2Cs). $PP2A_C$ consisted of the 36 kDa catalytic subunit (C36) only, whereas $PP2A_1$ contained equimolar amounts of the A (PR65), B (PR55) and C subunits. All expressed PP2C isoforms contained a single catalytic subunit of 42–45 kDa. The advantages of using the expressed catalytic domain of HMG-CoA reductase (HMG_C) as the substrate in this study were twofold: (i) it is phosphorylated stoichiometrically by AMPK at a single defined site (corresponding to Ser-871 in the complete sequence) [14]; (ii) phosphorylated HMG-CoA reductase is one of the best-known protein substrates for PP2C in vitro [21]. Using $PP2A_C$, there was an excellent correlation between removal of [^{32}P]phosphate from HMG_C and recovery of HMG-CoA reductase activity, and on extrapolation to 100% dephosphorylation the HMG-CoA reductase activity returned to that of a non-phosphorylated control (not shown).

3.2. Dephosphorylation of mutant HMG-CoA reductases by PP2A isoforms and PP2Cα

We compared the initial rates of dephosphorylation of various forms of HMG-CoA reductase which had point mutations at or around the phosphorylation site, Ser-871 (Fig. 2). In general the mutants were dephosphorylated at similar rates to the wild type, notable exceptions being: (i) $PP2A_C$ and

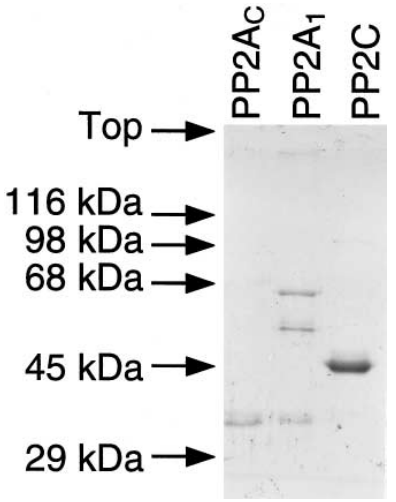

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified bovine heart $PP2A_C$ and $PP2A_1$, and human PP2C (α isoform). The gel was stained with Coomassie Blue, and the migration of molecular mass markers is shown at left.

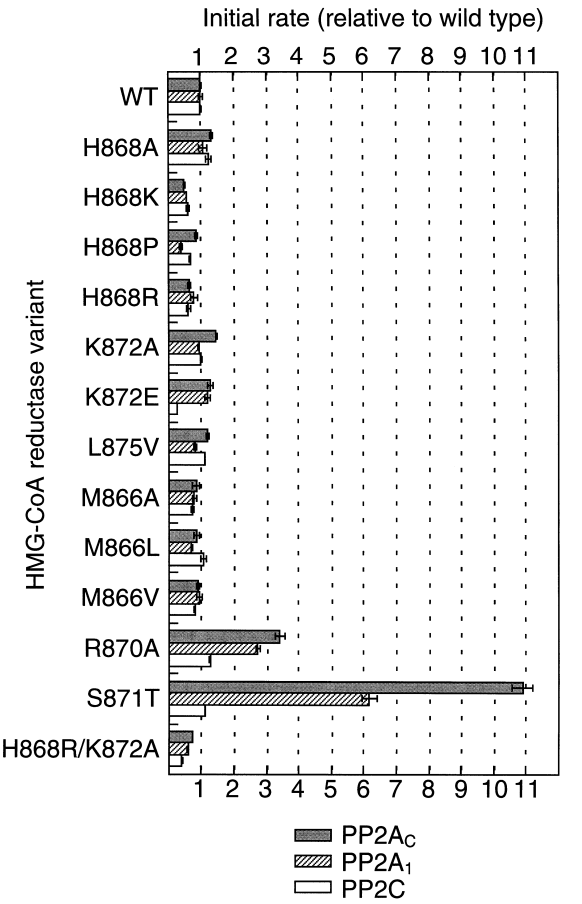

Fig. 2. Initial rate of dephosphorylation of HMG-CoA reductase variants by $PP2A_C$, $PP2A_1$ and human PP2Cα. The results are expressed relative to the rate obtained with wild-type HMG_C. A fixed concentration of HMG-CoA reductase (2.6 μM) was used in every case, and the results are the mean ± standard error of the mean for 3 separate experiments.

$PP2A_1$ (but not PP2Cα) greatly preferred phosphothreonine over phosphoserine (11-fold and 6-fold, respectively); (ii) $PP2A_C$ and $PP2A_1$ (but not PP2Cα) dephosphorylated the R870A mutant ≈3-fold more rapidly than the wild type; (iii) the K872E mutant was dephosphorylated 4-fold more slowly than the wild type by PP2Cα. In Fig. 2 the rates are expressed relative to the rates obtained using wild-type HMG-CoA reductase, and using this method of presentation the specificities of $PP2A_C$ and $PP2A_1$ appear very similar, although $PP2A_C$ did have a stronger preference for phosphothreonine over phosphoserine. However, if the results are expressed as absolute rates per mole of phosphatase (Table 1), it can be seen that $PP2A_C$ dephosphorylated all forms of HMG-CoA reductase much more slowly than $PP2A_1$ or PP2Cα. Using wild-type HMG_C, dephosphorylation by $PP2A_1$ was 87-fold faster, and by PP2Cα was 30-fold faster, than dephosphorylation by $PP2A_C$. On average, $PP2A_1$ dephosphorylated the 14 HMG-CoA reductase variants 73 ± 19-fold (mean ± standard deviation) faster than $PP2A_C$.

Attempts to measure more detailed kinetic parameters were not successful. With each of the three phosphatases and wild-type HMG_C as substrate, there was little sign of saturation of the rate at substrate concentrations as high as 50 μM. It was

not feasible to use the substrates at concentrations higher than this.

3.3. Dephosphorylation of mutant HMG-CoA reductases by mouse PP2Cα and five splice variants of PP2Cβ

Recently, cDNAs encoding the β isoform of mouse PP2C has been shown to exist as five variants which would give rise to distinct amino acid sequences (β1 through β5), apparently due to alternative splicing of a single gene product. To examine whether these isoforms exhibited any differences in substrate specificity, we used wild-type HMG_C and two of the mutants to examine their specificity. In Fig. 3 the results are expressed as moles phosphate released per mole PP2C per second. The results confirmed that human PP2C showed no preference for phosphothreonine over phosphoserine. Surprisingly, all six mouse isoforms did show a moderate preference for phosphothreonine, with S871T being dephosphorylated at a rate 2- to 4-fold faster than the wild type. Substitution of a glutamic acid for the lysine at the +1 position (K872E mutant) had a moderately deleterious effect on dephosphorylation by all human and mouse PP2C isoforms.

4. Discussion

In general, the results obtained in this study using native protein substrates based on HMG-CoA reductase are in qualitative agreement with those obtained previously using synthetic peptide substrates [11–13]. In most cases substitution of amino acids within five residues of the phosphoamino acid had only moderate effects on dephosphorylation by $PP2A_C$, $PP2A_1$ or PP2C. The most striking differences between wild-type and mutant HMG-CoA reductases are discussed below:

1. Both $PP2A_C$ (11-fold) and $PP2A_1$ (6-fold) exhibited marked preferences for HMG-CoA reductase containing phosphothreonine (Tp), rather than phosphoserine (Sp) in the same sequence context. A similar preference has been noted previously using synthetic peptide substrates with various forms of PP2A [11,12], and using a protein substrate (MAP kinase kinase, MAPKK1) with $PP2A_1$ [22].

2. Using the synthetic peptides RRATpVA and RRASpVA, rabbit PP2C (a mixture of α and β isoforms) has also been found to have a strong (30-fold) preference for phosphothreonine over phosphoserine [13]. Surprisingly, with HMG-CoA reductase variants as substrates human PP2Cα did not exhibit any such preference, although mouse PP2Cα and five isoforms of mouse PP2Cβ did exhibit a moderate (2- to 4-fold) preference. Whether these discrepancies represent genuine species differences, or whether they are connected with the method of expression and/or purification of the PP2C isoforms, is not clear.

Table 1
Initial rates of dephosphorylation of HMG-CoA reductase variants, expressed as mmoles phosphate released per mole of protein phosphatase per second at 30°C (mean ± standard error of the mean for three determinations)

Variant	PP2AC	PP2A1	PP2C
WT	2.67 ± 0.04	231 ± 12	81.0 ± 2.6
H868A	3.57 ± 0.11	248 ± 29	100.0 ± 8.4
H868K	1.31 ± 0.03	129 ± 2	49.0 ± 3.3
H868P	2.29 ± 0.15	90 ± 8	55.0 ± 1.5
H868R	1.72 ± 0.08	181 ± 25	50.0 ± 3.8
K872A	3.90 ± 0.08	212 ± 8	80.0 ± 1.4
K872E	3.41 ± 0.23	273 ± 18	21.0 ± 1.1
L875V	3.16 ± 0.12	188 ± 13	90.0 ± 0.8
M866A	2.27 ± 0.28	182 ± 11	60.0 ± 3.3
M866L	2.33 ± 0.23	161 ± 6	86.0 ± 6.7
M866V	2.39 ± 0.16	216 ± 21	64.0 ± 1.0
R870A	9.13 ± 0.40	631 ± 14	103.0 ± 2.1
S871T	29.10 ± 0.83	1424 ± 51	90.0 ± 1.2
H868R/K872A	1.91 ± 0.02	137 ± 5	32.0 ± 2.2

All variants were at a fixed substrate concentration of 2.6 μM. PP2C was the human α isoform.

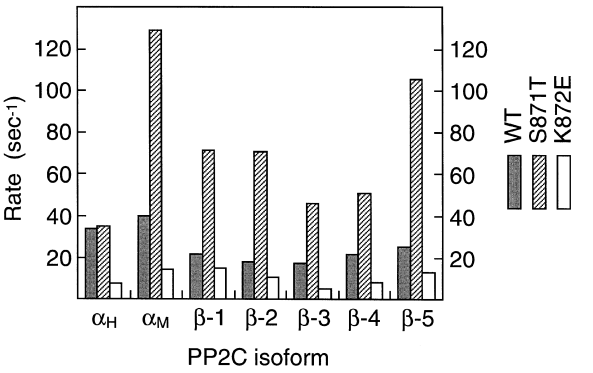

Fig. 3. Initial rate of dephosphorylation of the wild type and the S871T and K872E variants of HMGC by PP2C isoforms. The results are expressed as moles phosphate released per mole phosphatase per second at 30°C: this is not a true turnover number since a single arbitrary substrate concentration (2.6 μM) was used. α_H, α_M, human and mouse α isoforms; β-1...β-5, mouse β isoforms.

3. Replacement of the arginine at the P-1 position on HMG-CoA reductase with alanine (R870A) had no effect on human PP2Cα, but caused a ≈3-fold increase in the rate of dephosphorylation by both $PP2A_C$ and $PP2A_1$ (Fig. 2). A deleterious effect for PP2A of a basic residue at this position does not appear to have been reported previously. In the crystal structure for PP1γ, the active site where the phosphate group binds is located in a small pocket, and there are two basic residues (Arg-96 and Arg-221) on the outer edge of this pocket [23]. These arginines are conserved in PP2A, and it is possible that they contribute to the negative effect of an arginine at the P-1 position on the substrate.

4. Replacement of the lysine at the P+1 position by glutamic acid (K872E) had no significant effect on dephosphorylation by either form of PP2A, but reduced the rate of dephosphorylation by all forms of PP2C by up to 4-fold. This is consistent with previous findings that RRSTpEA was dephosphorylated >10-fold more slowly than RRSTpVA by a mixture of rabbit PP2Cα and PP2Cβ [13], although the effect was smaller with the native protein rather than the peptide substrate. The present work shows that it is the negative charge that has the deleterious effect, since there was no difference in the rate of dephosphorylation of the wild type and the K872A mutant. In the structure for human PP2Cα, the catalytic site is in a deep channel, the floor of which is lined with acidic residues, including Glu-37, Asp-239 and Asp-243 [24]. This may account for the inhibitory effect of an acidic residue located at the P+1 position on the substrate. HMG-CoA reductase may make more contacts with PP2C than short peptides, so that the inhibitory effect of an acidic residue at P+1 is less marked with the protein substrate.

One thing that is clear from this study is that the specific-

ities of both PP2A and PP2C are different from those of AMPK. Mutants of HMG-CoA reductase which do not have a bulky hydrophobic residue at the P-5 position (M866A) or a basic residue at the P-4 position (H868A) are very poor substrates for AMPK [14], but these changes are neutral with respect to dephosphorylation by PP2A or PP2C (Fig. 2).

The *relative* specificities of $PP2A_C$ and $PP2A_1$ for different HMG-CoA reductase variants (i.e. expressing the rates relative to those obtained with wild-type HMG-CoA reductase) are not markedly different (Fig. 2). However, the *absolute* rates of dephosphorylation are dramatically different (Table 1). $PP2A_1$ dephosphorylated all HMG-CoA reductase variants from 40- to 100-fold more rapidly than $PP2A_C$. Clearly the free catalytic subunit of PP2A is a very poor HMG-CoA reductase phosphatase, and the presence of the A and B subunits has a dramatic effect on the absolute rate, but not on the selectivity for different HMG-CoA reductase variants. Large effects of the A and/or B subunits on selectivity for specific protein substrates have been reported before (reviewed in [25]). Our results are consistent with the idea that the A and B subunits can have a marked effect on initial binding of a protein substrate to the phosphatase, although recognition of the area immediately around the phosphorylation site seems to be a function of the C subunit alone.

The different PP2C isoforms utilized in the present study do not differ markedly in specificity from one another. This is not perhaps surprising, since the mouse α and β are 80% identical within the catalytic domain, while the differences between the five mouse β isoforms all occur in the C-terminal region outside of the conserved catalytic domain [8].

Acknowledgements: This study was supported by a Programme Grant and a Studentship from the Wellcome Trust. We thank Nick Helps and Tricia Cohen for human PP2Cα, Steve Davies for AMPK, Bob MacKintosh for $PP2A_C$, Greg Moorhead for $PP2A_1$, and David Barford for helpful discussion.

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